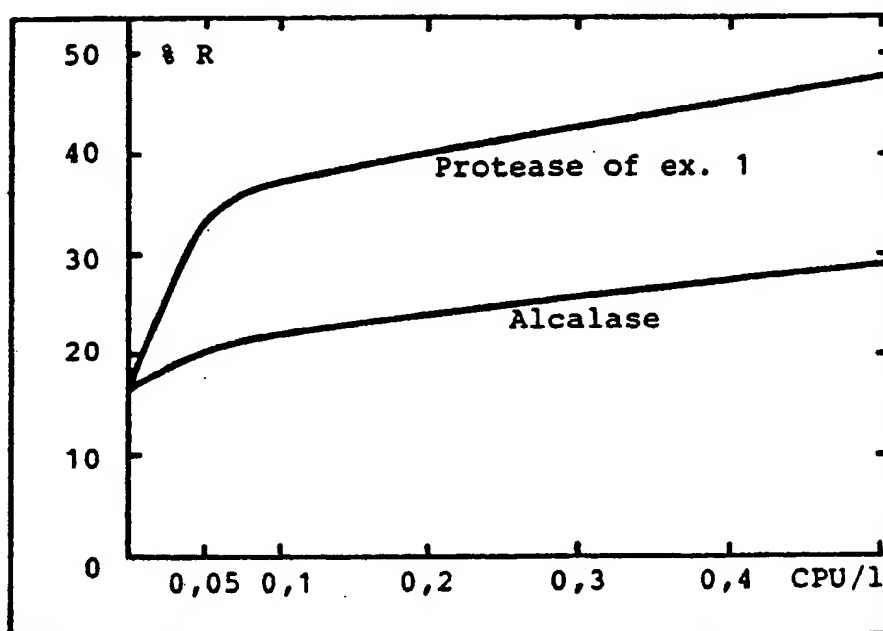




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(54) Title: ALKALINE PROTEASE DERIVED FROM BACILLES PRODUCTION AND USE THEREOF



(57) Abstract

Protease derived from a novel alkalophilic *Bacillus* species exhibits a remarkably enhanced stability towards bleaching agents of the peroxy type and is useful in detergents.

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Alkaline protease derived from *Bacillus* production
and use thereof

The present invention is directed to a novel alkaline protease preparation having enhanced oxidation stability, to a method for the preparation thereof, and to
5 its use as an additive for cleaning agents. Typical cleaning agents are detergent compositions for laundering.

BACKGROUND OF THE INVENTION

Proteolytic enzymes produced by cultivation of members of the genus *Bacillus* in suitable nutrient media are
10 widely used in detergent compositions. Examples of such commercial protease products are ALCALASE, ESPERASE and SAVINASE (these are registered trade marks), all supplied by NOVO Industri A/S, Denmark. These exhibit adequate storage stability in the presence of the usual components of
15 detergent compositions, such as sequestering agents, surfactants and even bleaching agents of the peroxy type, provided that they are suitably protected from their physical environment in the detergent composition, for example by their incorporation into suitably coated particles.

20 However, in the laundering process where the solubilized enzyme is exposed to the same agents in solution, stability may become critical in the sense that the enzyme is prone to destruction prior to the completion of its intended stain-removing action, particularly at elevated temperatures
25 in the presence of bleaching agents of the peroxy type, such as percarbonate, perborate, and persulfate salts. Since all *Bacillus*-derived proteases known in the art exhibit an appreciable instability at laundering temperatures exceeding 50°C in the presence of a peroxy compound it is often
30 recommended to prolong the washing programme by including a holding time at lower temperature when detergent protease compositions are used.

Attempts to enhance the stability of Bacillus derived serine proteases towards oxidizing agents by applying the technique of site specific mutation to the corresponding genes is known in the art, e.g. from European Patent 5 Publication No. 0 130 756 which discloses stabilized subtilisins by substituting the methionine residue next to the active-site-serine residue with amino acid residues which are less prone to oxidation. However, the obstacles associated with the adaption of recombinant DNA strategies to 10 large scale manufacturing processes are well-known, the most frequent ones being instability of the engineered microorganism through loss of genetic material and lower yields compared with those obtained from the wild strain or its conventional mutants.

15 As far as the inventors hereof are aware the prior art is silent about non-engineered Bacillus derived serine proteases exhibiting an enhanced stability towards oxidation as compared with commercial Bacillus proteases.

The object of the present invention is to overcome 20 the above-mentioned instability problems encountered with the Bacillus-derived alkaline protease preparations known heretofore. According to the invention this object has been met in that it has surprisingly been found that a number of Bacillus strains belonging to what is presently defined as 25 the same novel species produce alkaline protease which exhibits a remarkably enhanced stability towards bleaching agents of the peroxy type conventionally used for laundering.

Normally, the alkaline protease produced by the novel Bacillus species of this invention will contain three 30 main constituent, mutually distinguishable alkaline proteases (herein designated A, B and C) often in proportions, measured in protease activity units, of roughly one third of each. However, variant strains may lack the capability of producing at least one of these constituent proteases. Fortuitously, 35 all three constituent proteases show similar detergency and stability characteristics.

SUMMARY OF THE INVENTION

Thus, according to its first aspect the present invention provides a protease preparation comprising alkaline protease derived from a novel alkalophilic Bacillus species defined by the strains NCIB 12288 and NCIB 12289 which alkaline protease retains a residual activity measured in Casein Protease Units (CPU, vide infra) after 30 minutes at pH 9.5 in a solution of 1.75 g/l of sodium tripolyphosphate (STPP), 1.0 g/l of sodium perborate and 0.1 g/l of tetraacetythylenediamine (TAED) of at least 80% and 60% at temperatures of 40°C and 50°C, respectively, of the residual activity at 25°C in the absence of sodium perborate and TAED.

Another aspect of the present invention provides a protease preparation comprising at least 10% by activity in CPU of a constituent protease A which is immunochemically identical to the protease identified by the position of peak A in fig. 1 of the accompanying drawings and has the following additional characteristics: a molecular weight of about 41.5 kD, an isoelectric point of about 9.3, a specific activity of at least 150 Anson Units and 250 Hemoglobin Protease Units (AU and HPU, respectively, vide infra) per gm of protein, and a residual activity measured in CPU after 30 minutes at pH 9.5 in a solution of sodium tripolyphosphate (STPP, 1.75 g/l), sodium perborate (1.0 g/l), and tetraacetythylenediamine (TAED, 0.1 g/l) of at least 80% and 60% of temperatures of 40°C and 50°C, respectively, of the residual activity at 25°C in the absence of sodium perborate and TAED.

In a preferred embodiment the protease preparation is essentially devoid of proteases B and C, identified by peak B of fig. 1 and by peak C of fig. 1 and 2, respectively.

In yet another preferred embodiment of the invention the balance of the protease activity is provided jointly by said proteases B and C or singly by either of them. Immunochemically, proteases B and C are identical to

each other and different from protease A. Protease B and C have molecular weights of about 28.5 and 31 kD and isoelectric points of about 8.8 and 5.2, respectively.

The present invention further provides a protease
5 preparation comprising essentially said protease B.

In addition, the present invention is directed to a protease preparation comprising essentially said protease C.

According to a further aspect of this invention there is provided a method for producing a protease
10 preparation, which process is characterized by cultivating a strain of the novel Bacillus species defined by the strains NCIB 12288 and NCIB 12289 under aerobic conditions in a nutrient medium containing assimilable sources of carbon, nitrogen, and phosphorus, preferably followed by recovery of
15 the protease preparation from the fermentation broth.

In a preferred embodiment of producing the protease preparation the fermentation is conducted with a strain of the Bacillus species defined above, which strain is mutated so as to essentially block its capability of synthesizing one
20 or two of the constituent proteases whilst maintaining its capability of producing the remaining constituent protease or proteases.

In another preferred mode of producing the protease preparation the fermentation is conducted with a Bacillus
25 strain selected from the group consisting of NCIB 12288, NCIB 12289, NCIB 12512 and NCIB 12513.

According to yet another aspect there is provided a cleaning agent containing an effective amount, preferably at least 0.1 AU, more preferred from 0.5 to 10 AU, of the
30 protease preparation of this invention per 100 g of cleaning agent. The cleaning agent may be a powder or a liquid wherein the protease preparation is protected, for example by incorporation into granules or as a suspension in a hydrophobic medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the elution chromatogram of the protease preparation derived from NCIB 12289 on a CM Sepharose® CL 6B column, subsequent to gel filtration on 5 Sephadex® G 25.

Fig. 2 shows affinity chromatography of protease C on a bacitracin column.

Figs. 3 and 4 are exemplary of the pH-activity and temperature-activity curves, respectively, of the protease 10 preparation of this invention, all protease components exhibiting similar temperature- and pH-activity characteristics.

Figs. 5 and 6 illustrate the results of comparative washing experiments conducted with the protease preparation 15 of this invention and ALCALASE using two different detergents. Experimental conditions are given in Example 5 hereinafter.

Fig. 7 illustrates the results of washing experiments conducted with each of the constituent proteases.

20 DETAILED DESCRIPTION OF THE INVENTIONThe microorganism, taxonomy, morphology, biochemical reactionsThe microorganism:

The microorganisms productive of the alkaline 25 protease of the present invention were isolated essentially by the method for the selection of alkalophilic Bacilli described in British Patent No. 1,243,784. They will not grow on ordinary media for Bacillus strains unless these are supplemented by either 0.1 M carbonate buffer, pH 9.0 - 9.7 30 or a 7 - 10% (w/v) solution of NaCl, the 0.1 M carbonate buffer, pH 9.0 being the preferred supplement. Two cultures

have been deposited with The National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen, United Kingdom for the purposes of patent procedure on the date indicated below. NCIB, being an international depository 5 authorized under the Budapest Treaty of 1977, affords permanence of the deposit and accessibility thereto by the public in accordance with Rules 9 and 11, respectively, of the above treaty.

| | <u>Date of Deposit</u> | <u>Depositor's Reference</u> | <u>NCIB Designation</u> |
|----|------------------------|------------------------------|-------------------------|
| 10 | 8 July 1986 | C608 | 12288 |
| | 8 July 1986 | C609 | 12289 |
| | 5 August 1987 | C610 | 12512 |
| | 5 August 1987 | C611 | 12513 |

Taxonomy:

- 15 Medium TY was used for taxonomic investigations which were conducted according to the methods of Ruth Gordon et al. (The Genus Bacillus, Agriculture Handbook no. 427, U.S.D.A. 1973)

Composition of medium TY:

| | | |
|----|---------------------------------------|------|
| 20 | Trypticase | 20 g |
| | Yeast extract | 5 g |
| | FeCl ₃ .6H ₂ O | 7 mg |
| | MnCl ₂ .4H ₂ O | 1 mg |
| | MgSO ₄ .7H ₂ O | 15 g |
| 25 | distilled H ₂ O to 1000 ml | |

pH adjusted to 7.3 with KOH before autoclaving for 15 min at 121°C.

Liquid media for the determination of the production of acid from carbohydrates were supplemented with 10% NaCl before autoclaving.

Solid media were supplemented with 2% agar before autoclaving and sterile carbonate buffer pH 9.0 was aseptically added to a final concentration of 0.1 M just before pouring the slants.

The microorganisms of this invention are aerobic, spore forming bacteria belonging to the genus Bacillus.

10 Morphology: Vegetative rods with a diameter of 0.6 - 0.8 micron, length 1.2 - 3 micron.

Spores: Central to subterminal, oval, not swelling the sporangia.

| <u>Biochemical reactions:</u> | | <u>NCIB 12288</u> | <u>NCIB 12289</u> |
|-------------------------------|---------------------------------|-------------------|-------------------|
| 15 | Production of catalase | + | + |
| | Production of acetoin (VP-test) | - | - |
| | Production of indole | - | - |
| | Anaerobic growth | - | - |
| | Growth at 50°C | - | - |
| 20 | Growth in 7% NaCl | + | + |
| | Growth at pH 5.7 | - | - |
| | Hydrolysis of starch | + | + |
| | Hydrolysis of hippurate | - | - |
| | Hydrolysis of casein | + | + |
| 25 | Hydrolysis of tyrosine | + | + |
| | Deamination of phenylalanine | - | - |
| | Reduction of nitrate to nitrite | - | - |
| | Acid from glucose | + | + |
| | Acid from sucrose | + | + |
| 30 | Acid from xylose | + | - |
| | Acid from mannitol | (+) | (+) |

None of the strains NCIB 12288 and NCIB 12289 produced acid from arabinose, mannose, melibiose, raffinose, or sorbitol.

Optimal growth conditions are 30 - 37°C, pH 9.0.

The above taxonomic results show that the two strains do not belong to any previously described species, but should be considered to define a novel Bacillus species.

5 Taxonomic data for strains NCIB 12512 and NCIB 12513 are very similar to the above, indicating that all four strains belong to the same species.

Assay for proteolytic activity

OPA-Casein method

10 Proteolytic activity is determined with casein as the substrate. One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1 millimole of primary amino groups (determined by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30

15 minutes at 25°C and pH 9.5.

A 2% (w/v) solution of casein (Hammarsten, supplied by Merck A.G., West Germany) is prepared with the Universal Buffer described by Britton and Robinson (Journ.Chem.Soc. 1931, p. 1451), adjusted to pH 9.5.

20 Two ml of substrate solution is preincubated in a water bath for 10 minutes at 25°C. 1 ml of enzyme solution containing b g/ml of enzyme preparation, corresponding to about 0.2 - 0.3 CPU/ml of Britton-Robinson buffer (pH 9.5), is added. After 30 minutes of incubation at 25°C the reaction

25 is terminated by the addition of a stopping agent (5 ml of a solution containing trichloroacetic acid (17.9 g), sodium acetate (29.9 g), and acetic acid (19.8 g), filled up to 500 ml with deionized water). A blank is prepared in the same manner as the test solution, except that the stopping agent

30 is added prior to the enzyme solution.

The reaction mixtures are kept for 20 minutes in the water bath, whereupon they are filtered through Whatman® 42 paper filters.

Primary amino groups are determined by their colour development with o-phthaldialdehyde (OPA).

Disodium tetraborate decahydrate (7.62 g) and sodium dodecylsulfate (2.0 g) is dissolved in 150 ml of water. OPA (160 mg) dissolved in 4 ml of methanol is then added together with 400 μ l of beta-mercaptoethanol, whereafter the solution is made up to 200 ml with water.

To the OPA reagent (3 ml) is added 400 μ l of the above-mentioned filtrates with mixing. The optical density (OD) at 340 nm is measured after about 5 minutes.

The OPA test is also performed with a serine standard containing 10 mg of serine in 100 ml of Britton-Robinson buffer (pH 9.5). The buffer is used as a blank.

The protease activity is calculated from the optical density measurements by means of the following formula:

$$\text{CPU/ml of enzyme solution} = \frac{(\text{OD}_t - \text{OD}_b) \times C_{\text{ser}} \times Q}{(\text{OD}_{\text{ser}} - \text{OD}_B) \times \text{MW}_{\text{ser}} \times t_i}$$

$$20 \text{ CPU/g of enzyme preparation} = \text{CPU/ml} : b$$

wherein OD_t , OD_b , OD_{ser} and OD_B is the optical density of the test solution, blank, serine standard, and buffer, respectively, C_{ser} the concentration of serine in mg/ml in the standard, MW_{ser} the molecular weight of serine. Q is the dilution factor (in this instance equal to 8) for the enzyme solution, and t_i is the incubation time in minutes.

OPA-hemoglobin method

Proteolytic activity is determined with urea-denatured hemoglobin as the substrate. One Hemoglobin Protease Unit (HPU) is defined as the amount of enzyme liberating 1 millimole of primary amino groups (determined by

comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5.

A 2% (w/v) solution of urea-denatured hemoglobin is prepared with 0.2 M borate buffer, adjusted to pH 9.5.

Two ml of substrate solution is preincubated in a water bath for 10 minutes at 25°C. 1 ml of enzyme solution containing b g/ml of enzyme preparation, corresponding to about 0.2 - 0.3 HPU/ml of borate buffer, is added.

Incubation, termination of the reaction and determination of primary amino groups by means of the OPA reagent are the same as those described above for the OPA-casein method.

The OPA test is also performed with a serine standard containing 10 mg of serine in 100 ml of the borate buffer, the buffer itself serving as a blank.

The protease activity in HPU is calculated from a formula identical to that used in the OPA-casein method.

Modified Anson-hemoglobin method

In the Anson-hemoglobin method for the determination of proteolytic activity, denatured hemoglobin is digested under standard conditions. The undigested hemoglobin is precipitated with trichloroacetic acid (TCA) and the amount of TCA soluble product is determined with Folin-Ciocalteu phenol reagent.

One Anson Unit (AU) is the amount of enzyme which under standard reaction conditions digests hemoglobin at such an initial rate that there is liberated per minute an amount of TCA soluble product which gives the same colour with phenol reagent as one milliequivalent of tyrosine.

The standard reaction conditions are the following: Temperature 25°C; reaction time 10 min.; pH 7.5.

For further reference, see M.L. Anson, Journal of General Physiology vol. 22 (1939), pp. 79-89; O. Folin and V. Ciocalteu, The Journal of Biological Chemistry vol. 73 (1972), pp. 627-636.

Different values for proteolytic activity will invariably be obtained depending on the choice of analytical method. While the OPA-casein assay is convenient for monitoring proteolytic activity for laboratory and production purposes, it may be said that proteolytic activity towards a porphyrin containing substrate, such as hemoglobin, is more relevant for assessing the utility of a detergent protease because of the generally experienced difficulties with removal of stains from blood and chlorophyll. A high specific activity of a detergent protease towards porphyrin containing substrates is desirable.

The protease preparation

The Bacillus strains productive of the protease of this invention are cultivated under aerobic conditions in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients, the medium being composed in accordance with the principles of the known art.

Suitable carbon sources are carbohydrates, such as sucrose, glucose and starch, or carbohydrate containing materials such as cereal grains, malt, rice and sorghum. Polysaccharides are usually at least partially converted into fermentable sugars by suitable hydrolyzing enzymes prior to the fermentation process. The carbohydrate concentration incorporated in the medium may vary widely, e.g. up to 25% and down to 1 - 5%, but usually 5 - 15% will be suitable, the percentages being calculated as equivalents of glucose.

The nitrogen source in the nutrient medium may be of inorganic and/or organic nature. Suitable inorganic nitrogen sources are nitrates and ammonium salts. Among the organic nitrogen sources quite a number are regularly used in fermentation processes involving the cultivation of bacteria. Illustrative examples are soybean meal, cotton seed meal, peanut meal, casein, corn steep liquor, yeast extract, urea, and albumin. In addition, the nutrient medium should also contain the usual trace substances.

The optimum temperature for propagation of the Bacillus strains and for their production of protease is routinely ascertained by the skilled artisan. The cultivation may be conducted in the temperature range of from 25°C to 5 35°C, preferably at alkaline pH values. The latter may be obtained by addition of suitable buffers, such as sodium carbonate or mixtures of sodium carbonate and sodium bicarbonate after sterilization of the growth medium. For cultivation in tank fermentors it is necessary to use 10 artificial aeration. The rate of aeration is similar to that used in conventional tank fermentation.

After fermentation, liquid enzyme concentrates may be produced by removal of coarse material from the broth and, if desired, concentration by evaporation at low temperature 15 or by reverse osmosis. Finally, preservatives may be added to the concentrate.

Solid enzyme preparations may be prepared from the purified and/or concentrated broth by precipitation with salts, such as Na_2SO_4 or with water miscible solvents, such 20 as ethanol or acetone; removal of the water in the broth by suitable drying methods such as spray-drying may also be employed.

The proteolytic activity of the protease preparation so obtained is usually in the range of from 0.1 25 to 10 CPU/g.

The pH-activity and temperature-activity curves of protease according to this invention are shown in fig. 1 and 2. The sample used was that prepared in example 1 below, and activity was determined by the CPU method described above, 30 except that pH or temperature was changed. The curves show that optimum pH with casein as substrate is about 6 to 11, and that optimum temperature for 30 minutes reaction is about 50-60°C.

Purification of Constituent Proteases

The first, second and third protease (hereinafter referred to as protease A, Protease B and protease C, respectively) may be separated and purified by separation
5 techniques generally known by the skilled artisan.

An initial purification of the crude protease may be effected by dissolution, e.g. in a suitable buffer, followed by protein precipitation, e.g. with a salt, such as $(\text{NH}_4)_2\text{SO}_4$, or a water-miscible organic solvent, for example
10 acetone.

Further purification of the recovered precipitate is usually attained by chromatographic methods, for example including gel filtration in a buffer system as the initial step. Protease containing fractions of the eluate may be
15 located by monitoring U.V. absorbancy and/or by determining the protease activity.

Following gel filtration further purification is usually achieved by ion exchange chromatography, for example on a SEPHADEX® anion or cation exchanger, whereby at least
20 partial resolution of the constituent proteases may be effected, by using suitable buffer-salt-gradient systems. Rechromatography of peak material therefrom by means of the same or a different ion exchanger usually affords the protease species in a sufficiently pure state. If required,
25 further purification of the individual proteases may be achieved by subjecting them to affinity chromatography, for example on a bacitracin column.

Purification of the constituent proteases is conveniently monitored by gel electrophoresis, for example in
30 a polyacrylamide gel (PAGE) with or without sodium dodecylbenzenesulphonate (SDS). Autodigestion of the proteases may be prevented by active site blocking prior to electrophoresis, for example with phenylmethylsulphonyl fluoride (PMSF).

35 SDS-PAGE and isoelectric focusing (IEF) in the presence of marker proteins are convenient means for molecular weight (MW) and isoelectric point (pI)

determinations, respectively. According to these methods, proteases A, B and C have molecular weights of about 41.5, 28.5 and 31.0 kD, and isoelectric points of about 9.3, 9.2 and 5.2, respectively. Protease A is further characterized by 5 specific activities per gram of protein of about 180 AU/g and 270 HPU/g.

Immunochemical Characterization

Monospecific antisera against each of the purified proteases may be raised for example by immunizing rabbits 10 according to the regimen described by N. Axelsen et al. in: A manual of Quantitative Immuno-electrophoresis, Blackwell Scientific Publications, 1973, chapter 23. Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation $((\text{NH}_4)_2\text{SO}_4)$, followed by 15 dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex.

Immunochemical characterization of proteins is usually conducted either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental 20 Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706) or by crossed immunoelectrophoresis (N. Axelsen et al., supra, chapters 3 and 4).

Proteases B and C of the invention show 25 immunochemical identity to each other, and both show non-identity to protease A. Proteases A, B and C of the invention all show immunochemical non-identity to the following known alkaline Bacillus proteases:

- Alcalase (product of Novo Industri A/S, Denmark), derived 30 from Bacillus licheniformis, with Subtilisin Carlsberg as the active component.

- Savinase and Esperase (products of Novo Industri A/S), prepared from alkalophilic Bacillus sp. according to US 3,723,250.
 - API-21 (U.S. patent 4,480,037).
- 5 - The protease of Danish patent application No. DK 87/0544.

Cleaning agent

Cleaning agent according to the invention is typically a laundry detergent in liquid or powder form. In the case of a liquid detergent, the stability of the protease
10 during storage may be unsatisfactory, and it is therefore preferred to include enzyme stabilizers in the formulation. Thus, the liquid formulation may include polyols such as 1,2-propanediol (propylene glycol), typically 1 - 20%. Optionally, the formulation may include boric acid (or
15 alternatively, boric oxide, borax or a borak), typically up to 10%. Also optionally, formate or a salt of other lower fatty acid may be included, e.g. in an amount up to 10%. The calcium content in the liquid detergent may be from 0.001 to 0.1% (all %'es by weight).

20 Example 1

Protease preparation derived from strain NCIB 12289

Cells of Bacillus strain NCIB 12289 were harvested after 2 days' incubation at 30°C from slants of TY-agar supplemented aseptically with 0.1 M carbonate buffer pH 9.0
25 and 1% skim milk. The cells were transferred to 500 ml baffled shake flasks containing 100 ml BP-X medium + 0.1 M carbonate buffer pH 9.0.

Medium BP-X:

| | | |
|---|--|-------|
| | potato starch | 100 g |
| | barley flour | 50 g |
| | soy bean flour | 20 g |
| 5 | Na-caseinate | 10 g |
| | Na ₂ HPO ₄ ·12H ₂ O | 9 g |
| | TERMAMYL® 60L (NOVO) | 0.1 g |
| | PLURONIC TM | 0.1 g |
| | tap water to 1000 ml | |

10 The mixture was slowly heated from 60°C to 85°C during 30 minutes with agitation in order to liquefy the starch. The mixture was then heated quickly and kept above 95°C for 10 minutes before it was cooled, distributed into the shake flasks and autoclaved for 40 minutes at 121°C.

15 The inoculated shake flasks were incubated for 4 days at 25°C on a rotary shaker at 240 rpm. The fermentation broth (2.5 l, 54 CPU/l, as assayed by the OPA-casein method) was centrifuged for 30 minutes at 4000 rpm. The centrifuged liquid (2.0 l) was concentrated to 1.0 l by ultrafiltration
20 on a Mini-lab module from DDS, RO-Division, Denmark. After the concentration it was washed with 5.0 l of 0.01 molar sodium borate, pH 9.0, and finally concentrated further to 400 ml. It was then lyophilized, giving 25.5 g of crude preparation with an activity of 3.0 CPU/g. Yield: 57%.

Example 2Separation of constituent proteases A, B and C

Fermentation with NCIB 12289 was performed essentially as in Example 1. The culture broth (75 AU/kg) was
5 purified through the following steps:

- flocculation
- centrifugation
- evaporation
- blank filtration
- 10 - salt precipitation with Na_2SO_4
- redissolution
- blank filtration
- sterile filtration
- concentration by ultrafiltration and washing
- 15 - lyophilization

The lyophilized crude preparation (activity 15.3 CPU/g or 24.5 AU/g) was purified and separated into constituent proteases by the following sequence of steps.

1. Redissolution

- 20 2.5 g of protease was dissolved in 50 ml of the following buffer:
- dimethylglutaric acid 0.01 M
 - boric acid 0.1 M
 - calcium chloride 0.002 M
 - 25 sodium hydroxide to pH 7.0

2. Centrifugation

The redissolved protease was centrifuged at 18000 rpm at 25 - 30°C for 20 minutes.

3. Filtration

The supernatant was filtered on Whatman glassfilter type GF/F, 42 ml of clarified protease solution was collected.

5 4. Gelfiltration of Sephadex G 25 medium

40 ml of clarified protease solution was applicated to a G 25 column (5 cmØ x 22.6 cm) equilibrated with the buffer used in step 1. 10 ml fractions were collected, with a flow rate of 3.3 ml/min., and tested for activity. Fractions 10 19 - 25, comprising 54% of the applicated activity, were pooled, diluted to 100 ml and filtered on Millipore 0.45 µ filter type HA.

5. Ion exchange chromatography on CM Sepharose CL 6B

The filtered eluate was applicated on a CM 15 Sepharose CL 6B column (5 cmØ x 7.8 cm) equilibrated with the buffer used in step 1. Protease C and colored substances were washed out of the column with the same buffer. The other components were eluted with a 0 - 0.05 M NaCl gradient in 2 l of the same buffer. 10 ml fractions were collected, with a 20 flow rate of 3.3 ml/min., and monitored by UV-absorbance. The gradient was started after fraction 37. The UV-absorbing fractions 5 - 40 and 71 - 150 were tested for protease activity. The protease activity was found in 3 distinctive peaks, as seen in fig. 1.

25 Fractions 9 - 25, comprising 23.1% of the applicated activity, were pooled (Protease C).

Fractions 76 - 98, comprising 25.3% of the applicated activity, were pooled and designated Protease A.

30 Fractions 122 - 140, comprising 23.7% of the applicated activity, were pooled and designated Protease B.

6. Affinity chromatography on a bacitracin column

The column (1.6 cmØ x 12 cm) was equilibrated with the buffer of step 1. The pool of fractions 9 - 25 were applicated on the column. The protease was eluted with the same buffer with added NaCl (1 M) and isopropanol (25%). The flow rate was 5 ml/min. and the fraction size 10 ml. The elution diagram is shown in fig. 2 (OD at 280 nm vs. time). Fractions 24 - 27 comprising 73% of the applicated protease activity, were pooled and designated Protease C.

10 Example 3

Protease preparation derived from strain NCIB 12288

The same procedure as in Example 1 above was followed for the cultivation of strain NCIB 12288. The fermentation broth (0.3 l, 35 CPU/l) gave 11.7 g of crude preparation with an activity of 0.39 CPU/g. Yield: 43%.

Example 4

Protease preparation derived from strain NCIB 12512

The same procedure as in Example 1 above was followed for the cultivation of strain NCIB 12512. The fermentation broth (1.8 l, 30 CPU/l) gave 19.1 g of crude preparation with an activity of 1.0 CPU/g. Yield: 35%.

Example 5Protease preparation derived from strain NCIB 12513

The same procedure as in Example 1 above was followed for the cultivation of strain NCIB 12513. The resulting preparation was separated as in Example 2 above and was found to be devoid of protease B.

Example 6Stability of Bacillus proteases in solutions of STPP, sodium perborate and TAED

Solutions, each with an activity of 0.2 CPU per litre of the protease and containing 1.75 g/l of sodium tri-poly phosphate (STPP), 1.0 g/l of sodium perborate and 0.1 g/l of tetraacetyl- ethylenediamine (TAED) were incubated for 30 minutes at 40°C and 50°C. The residual activity was then measured according to the OPA-casein method, the activity after incubation at 25°C in the absence of sodium perborate and TAED being calculated as 100%. The results are presented in the following table:

| Protease | Percent residual activity after 30 minutes at | |
|-------------------------|--|---------|
| | 40°C | 50°C |
| ALCALASE | 55 - 70 | 5 - 17 |
| ESPERASE | 55 - 65 | 20 - 45 |
| SAVINASE | 40 - 50 | 0 - 10 |
| Protease of Example 1 | 90 - 100 | 60 - 75 |
| Protease of Example 3 | 95 - 100 | 70 |
| Protease A of Example 2 | 91 | 65 - 70 |

Example 7Washing Trials with Crude Protease Preparation

Washing tests were performed in a Terg-O-tometer (as described in Jay C. Harris: "Detergency Evaluation and Testing", Interscience Publishers Ltd. (1954), p. 60-61) at an agitation rate of 100 rpm for 10 minutes at 60°C with EMPA 116 test fabric swatches, 5 cm x 10 cm (cotton soiled with blood, milk, and carbon black), supplied by Eidgenössische Materialprüfungs- und Versuchsanstalt, Sankt Gallen, Switzerland. The following detergent compositions A and B were used in water of 9° German Hardness:

| | <u>Detergent A (g/l)</u> | <u>Detergent B (g/l)</u> |
|------------------|--------------------------|--------------------------|
| LAS | 1.0 | 1.0 |
| Soap | 0.1 | 0.1 |
| 15 STPP | 1.25 | 1.25 |
| Sodium silicate | 0.25 | 0.25 |
| Sodium sulphate | 2.4 | 2.4 |
| Sodium perborate | none | 1.0 |
| TAED | none | 0.1 |
| 20 pH | 9.5 | 9.5 |

wherein

LAS is linear alkylsulfonate (NANSA 80S, produced by Albright & Wilson)

STPP is sodium tripolyphosphate

25 TAED is tetraacetylenediamine.

The cloth to liquid ratio was 9 swatches per 900 ml of detergent solution. The detergent enzymes used were the enzyme preparation of Example 1 and ALCALASE protease, both at dosage levels of 0.05, 0.1 and 0.5 CPU/l.

30 The results of duplicate washing tests with detergents A and B are shown in Fig. 5 and 6, respectively, wherein the washing efficiency is expressed as medium values

of % Remission as determined by remission photometry by using a ZEISS ELREPHO photometer at 460 nm. The figure shows that the washing effect of the protease of this invention at dosage levels of about 0.05 CPU/l and above exceeds that of the ALCALASE protease both in the absence (Detergent A) and presence (Detergent B) of perborate + TAED.

Example 8

Washing trials with constituent proteases A, B and C

Washing tests were preformed in a Terg-O-tometer at 10 100 rpm for 10 min at 50°C. The following detergent composition was used in water of 9° German hardness:

| | | |
|----|------------------|----------|
| | LAS | 0.4 g/l |
| | AE | 0.15 g/l |
| | Soap | 0.15 g/l |
| 15 | STPP | 1.75 g/l |
| | Sodium silicate | 0.4 g/l |
| | CMC | 0.05 g/l |
| | EDTA | 0.01 g/l |
| | Sodium sulphate | 2.1 g/l |
| 20 | Sodium perborate | 1.0 g/l |
| | TAED | 0.1 g/l |
| | pH | 9.5 |

wherein

LAS, STPP and TAED are as in Example 7.

25 AE is alcohol ethoxylate (Berol 065, produced by Berol Kemi AB, Sweden).

Clean swatches consisted of white cotton textile, which was desized with amylase (BAN Novo), dried and cut in pieces (10 x 20 cm).

Clean swatches were immersed in spinach juice, squeezed between two rollers and dried in a tumbler at 60°C. This was repeated twice to obtain soiled swatches.

The cloth to liquid ratio was 8 swatches (4 clean 5 and 4 soiled) in 800 ml of detergent solution. Protease A, B and C from Example 2 were tested at dosages of 0.1, 0.5 and 1.0 CPU/l. The results are shown in fig. 7 as %. Remission at 460 nm versus protease dosage.

The figure shows that all three constituent 10 proteases have a good washing effect in detergent with perborate at 50°C.

Example 9Storage stability in liquid detergents

Liquid detergents were formulated as follows
(amounts in g):

| 5 | | A1/2 | B1/2 | C1/2 | D1/2 |
|----|---------------------------------------|-----------|-----------|-----------|-----------|
| | <u>Fraction I:</u> | | | | |
| | Water | 30.0/42.0 | 30.0/42.0 | 5.0/17.0 | 28.0/40.0 |
| | TEA | 10.0 | 10.0 | 10.0 | 10.0 |
| | CaCl ₂ , 2H ₂ O | 0.10 | 0.10 | 0.10 | 0.10 |
| 10 | DTPA | 0.10 | 0.10 | 0.10 | 0.10 |
| | DG | - | - | - | - |
| | Tri-sodium Citrate | 5.0 | 5.0 | 5.0 | 5.0 |
| | H ₃ BO ₃ | 0/5.0 | 0/5.0 | 0/5.0 | 0/5.0 |
| | <u>Fraction II:</u> | | | | |
| 15 | MPG | 0/12.5 | 0/12.5 | 0/12.5 | 0/12.5 |
| | Ethanol, 96% | 7.0 | 7.0 | 7.0 | 7.0 |
| | Oleic acid | 5.0 | 5.0 | 5.0 | 5.0 |
| | AE | 15.0 | 15.0 | 15.0 | 15.0 |
| | AES | 25.0 | 25.0 | - | 25.0 |
| 20 | LAS | - | - | 50.0 | - |
| | 10 N NaOH to pH: | 9.0 | 10.0 | 9.0 | 9.0 |
| | Water added to (g): | 100/129.7 | 100/129.7 | 100/129.7 | 100/129.7 |

TEA stands for triethanolamine, DTPA for penta-sodium salt of diethylene-triamine-pentaacetate, DG for sodium diglycolate, MPG for mono-propylene glycol, AES for alcohol-ethoxy sulphate (Dobanol 25-3S/60, product of Shell), LAS for linear alkyl-benzene sulphonate (Nansa 1169P), and AE stands for alcohol ethoxylate (Berol 160, product of Berol Kemi AB, Sweden). In each case, the ingredients in fraction I and II were mixed, and then fraction II was added slowly to fraction I while stirring.

The water content (including water contained in other ingredients) was about 41% w/w in all detergents.

The following protease preparations were tested:

- The lyophilized crude preparation of Example 2, dissolved
5 in a mixture of water and MPG (1:1 by weight) and diluted to an activity of 2.5 AU/g.
- Alcalase 2.5L (product of Novo Industri A/S, Denmark), a commercial liquid preparation of alkaline protease from Bacillus licheniformis.
- 10 - Esperase 8.0L (product of Novo Industri A/S, Denmark), a commercial liquid preparation of alkaline Bacillus protease prepared according to US 3,723,250.

The calcium content of each protease preparation was adjusted to 1.0% w/w (as Ca^{++}) by addition of CaCl_2 ,
15 $2\text{H}_2\text{O}$. In each experiment, 1% w/w of a protease preparation (after Ca^{++} adjustment) was added to one of the above detergents, the mixture was incubated at 50°C, and samples were taken after 0, 23, 47 and 134 hours incubation. The protease activity of each sample was determined, and the
20 results are shown below, expressed as residual activity in % of initial activity (i.e. activity after 0 days). pH was measured after 0 and 134 hours.

pH:

| | 0 h | 134 h | 0 h | 134 h |
|--------------|------|-------|-----|-------|
| Detergent | A1 | | A2 | |
| Ex. 2 | 9.0 | 9.0 | 9.0 | 9.0 |
| 5 ESPERASE | 9.0 | 9.1 | 9.0 | 9.0 |
| ALCALASE | 9.0 | 9.1 | 8.9 | 9.0 |
| Detergent | B1 | | B2 | |
| Ex. 2 | 10.0 | 9.9 | 9.9 | 9.9 |
| ESPERASE | 10.1 | 9.8 | 9.9 | 9.8 |
| 10 ALCALASE | 10.1 | 9.8 | 9.9 | 9.8 |
| Detergent | C1 | | C2 | |
| Ex. 2 | 9.2 | 9.2 | 9.0 | 9.0 |
| ESPERASE | 9.2 | 9.2 | 9.0 | 9.0 |
| ALCALASE | 9.2 | 9.2 | 9.0 | 9.1 |
| 15 Detergent | D1 | | D2 | |
| Ex. 2 | 9.1 | 9.2 | 9.1 | 9.0 |
| ESPERASE | 9.2 | 9.1 | 9.1 | 9.0 |
| ALCALASE | 9.2 | 9.1 | 9.1 | 9.0 |

% residual activity:

| | 23 h | 47 h | 134 h | 23 h | 47 h | 134 h |
|--------------|------|------|-------|------|------|-------|
| Detergent | | A1 | | | A2 | |
| Ex. 2 | 46 | 37 | 16 | 102 | 90 | 86 |
| 5 ESPERASE | 3 | 2 | 1 | 72 | 77 | 53 |
| ALCALASE | < 1 | < 1 | < 1 | 1 | 1 | < 1 |
| Detergent | | B1 | | | B2 | |
| Ex. 2 | 43 | 33 | 12 | 91 | 80 | 55 |
| ESPERASE | 2 | 2 | < 1 | 67 | 70 | 39 |
| 10 ALCALASE | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 |
| Detergent | | C1 | | | C2 | |
| Ex. 2 | 38 | 31 | 16 | 89 | 80 | 66 |
| ESPERASE | 2 | 2 | 21 | 73 | 72 | 41 |
| ALCALASE | < 1 | < 1 | < 1 | 15 | 13 | 3 |
| 15 Detergent | | D1 | | | D2 | |
| Ex. 2 | 46 | 37 | 19 | 92 | 87 | 75 |
| ESPERASE | 9 | 5 | 1 | 90 | 88 | 66 |
| ALCALASE | < 1 | < 1 | < 1 | 4 | 4 | 1 |

pH was nearly constant in all experiments. It is seen that protease of the invention is more stable than Esperase and Alcalase in all the detergent formulations tested. The results further show that borate and MPG are very effective as stabilizers for protease of the invention in liquid detergents.

International Application No: PCT/

/

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 6, line 10 of the description.**A. IDENTIFICATION OF DEPOSIT**Further deposits are identified on an additional sheet ☐ **Bacillus sp.nov.**

Name of depositary institution *

National Collection of Industrial Bacteria (NCIB)

Address of depositary institution (including postal code and country) *

c/o the National Collection of Industrial and Marine
Bacteria Ltd. Torry Research Station, P.O. Box 31,
135 Abbey Road, Aberdeen AB9 8DG, United Kingdom

Date of deposit *

8 July 1986 (08.07.86)

Accession Number *

NCIB 12288

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

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The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g. "Accession Number of Deposit")

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MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 6, line 11 of the description:**A. IDENTIFICATION OF DEPOSIT:**Further deposits are identified on an additional sheet ☐ **Bacillus sp.nov.**

Name of depository institution:

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International Application No: PCT/

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MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 6, line 12 of the description.**A. IDENTIFICATION OF DEPOSIT**Further deposits are identified on an additional sheet ☐ **Bacillus sp.nov.**

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Date of deposit:

5 August 1987 (05.08.87)

Accession Number:

NCIB 12512

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International Application No: PCT/

/

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 6, line 13 of the description.**A. IDENTIFICATION OF DEPOSIT**Further deposits are identified on an additional sheet ☐ **Bacillus sp.nov.**

Name of depository institution:

National Collection of Industrial Bacteria (NCIB)

Address of depository institution (including postal code and country):

c/o the National Collection of Industrial and Marine
Bacteria Ltd. Torry Research Station, P.O. Box 31,
135 Abbey Road, Aberdeen AB9 8DG, United Kingdom

Date of deposit:

5 August 1987 (05.08.87)

Accession Number:

NCIB 12513

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C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (If the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS** (Leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later. (Specify the general nature of the indications e.g. "Accession Number of Deposit")

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(Authorized Officer)

CLAIMS

1. A protease preparation comprising alkaline protease derived from a novel alkalophilic Bacillus species defined by the strains NCIB 12288 and NCIB 12289 which
5 alkaline protease retains a residual activity measured in Casein Protease Units (CPU) after 30 minutes at pH 9.5 in a solution of 1.75 g/l of sodium tripolyphosphate (STPP), 1.0 g/l of sodium perborate and 0.1 g/l of tetraacetythylenediamine (TAED) of at least 80% and 60% at
10 temperatures of 40°C and 50°C, respectively, of the residual activity at 25°C in the absence of sodium perborate and TAED.

2. A protease preparation comprising at least 10% by activity in CPU of a constituent protease A which is immunochemically identical to the protease identified by the
15 position of peak A in fig. 1 of the accompanying drawings and has the following additional characteristics: a molecular weight of about 41.5 kD, an isoelectric point of about 9.3, a specific activity of at least 150 Anson Units and 250 Hemoglobin Protease Units (AU and HPU, respectively) per gm
20 of protein, and a residual activity measured in CPU after 30 minutes at pH 9.5 in a solution of sodium tripolyphosphate (STPP, 1.75 g/l), sodium perborate (1.0 g/l), and tetraacetythylenediamine (TAED, 0.1 g/l) of at least 80% and 60% of temperatures of 40°C and 50°C, respectively, of
25 the residual activity at 25°C in the absence of sodium perborate and TAED.

3. The protease preparation of claim 2 which is essentially devoid of other proteases than said first protease A.

30 4. The protease preparation of claim 2, wherein the balance of the protease activity is provided jointly by proteases B and C or singly by either of these, said proteases being identified by peak B of fig. 1 and by peak C of fig. 1 and 2, by molecular weights of about 28.5 and 31.0
35 kD by isoelectric points of about 8.8 and 5.2, respectively, said proteases B and C being immunochemically identical to each other, but different from said protease A.

5. A protease preparation comprising essentially said protease B.

6. A protease preparation comprising essentially said protease C.

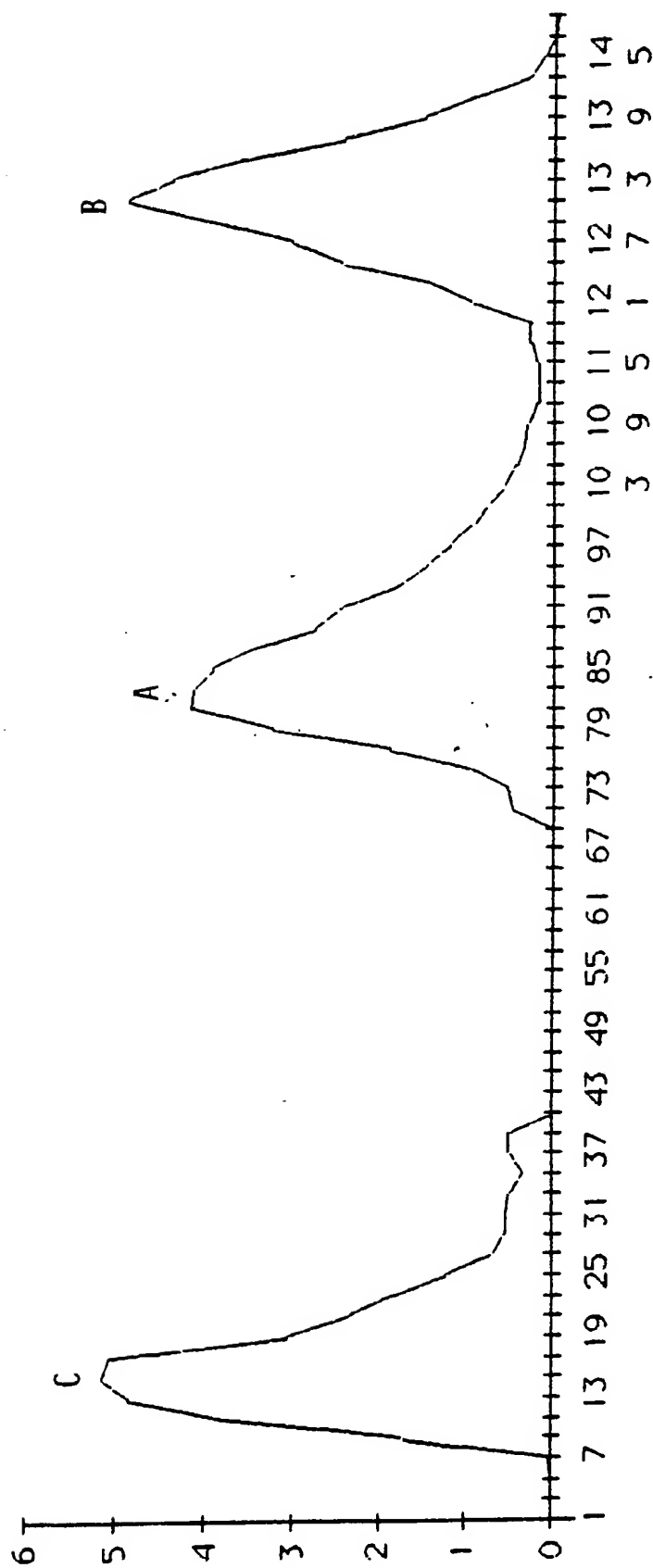
5 7. A method for producing a protease preparation, which process is characterized by cultivating a strain of the novel Bacillus species defined by the strains NCIB 12288 and NCIB 12289 under aerobic conditions in a nutrient medium containing assimilable sources of carbon, nitrogen, and
10 phosphorus, preferably followed by recovery of the protease preparation from the fermentation broth.

8. The method of claim 7, wherein the fermentation is conducted with a strain of the Bacillus species defined above, which strain is mutated so as to essentially block its
15 capability of synthesizing one or two of the constituent proteases whilst maintaining its capability of producing the remaining constituent protease or proteases.

9. The method of claim 7, wherein the fermentation is conducted with a Bacillus strain selected from the group
20 consisting of NCIB 12288, NCIB 12289, JP 75 and JP 170.

10. A cleaning agent containing an effective amount, preferably at least 0.1 AU, more preferred from 0.5 to 10 AU, of the protease preparation of any one of the claims 1 to 6 and/or protease preparation produced by the
25 method of any one of the claims 7 to 9.

Activity



Fraction

FIG. 1

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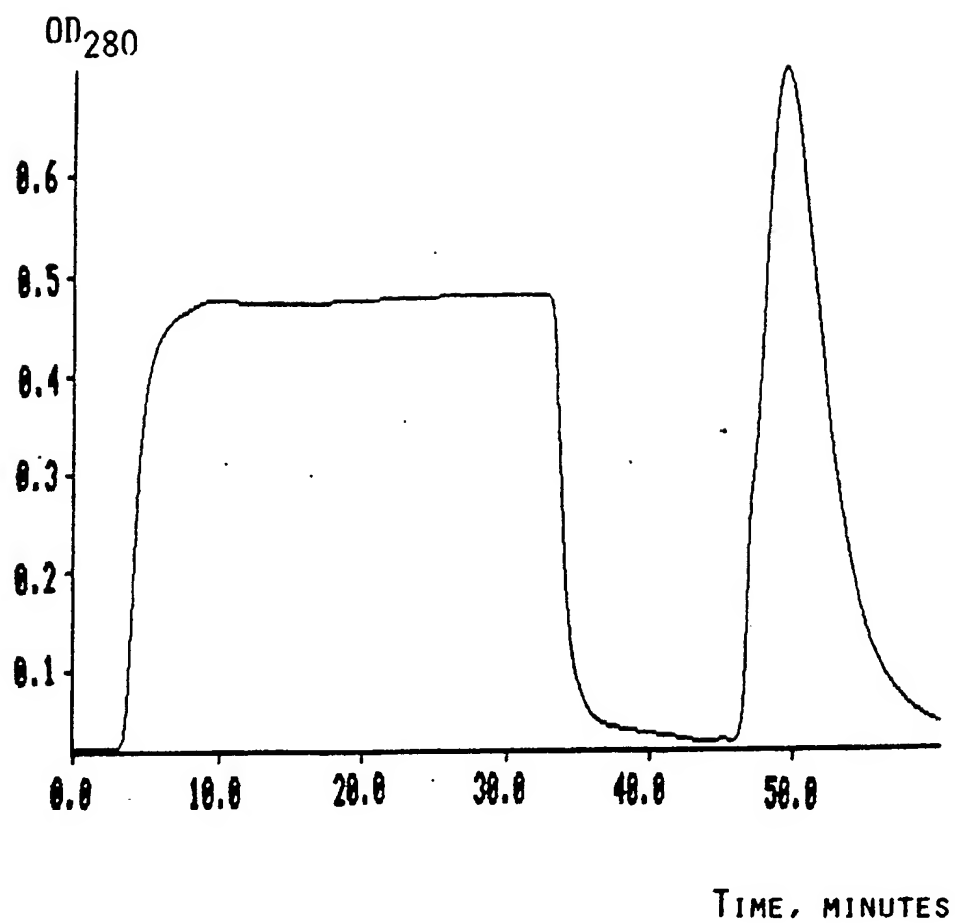


FIG. 2

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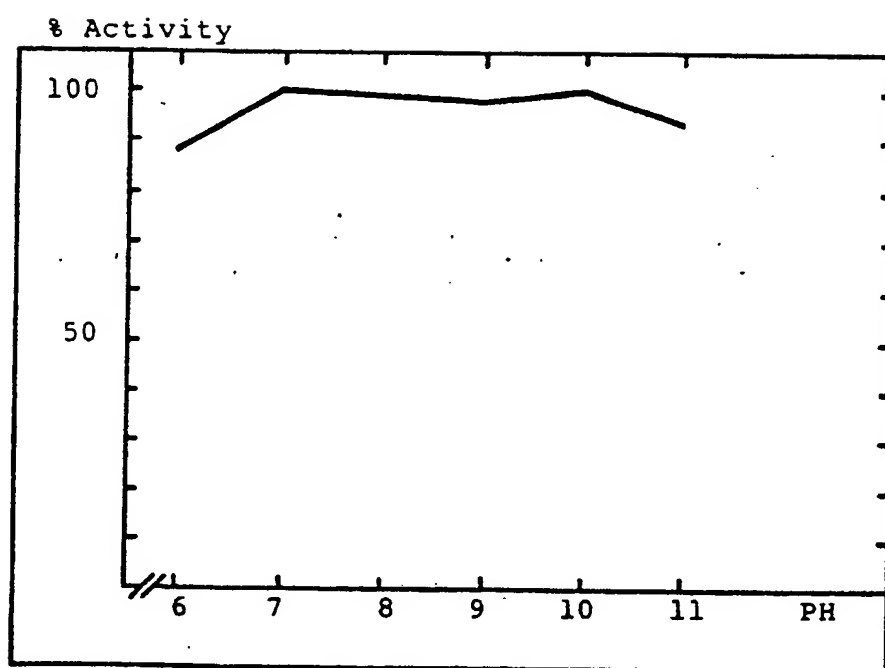


FIG. 3

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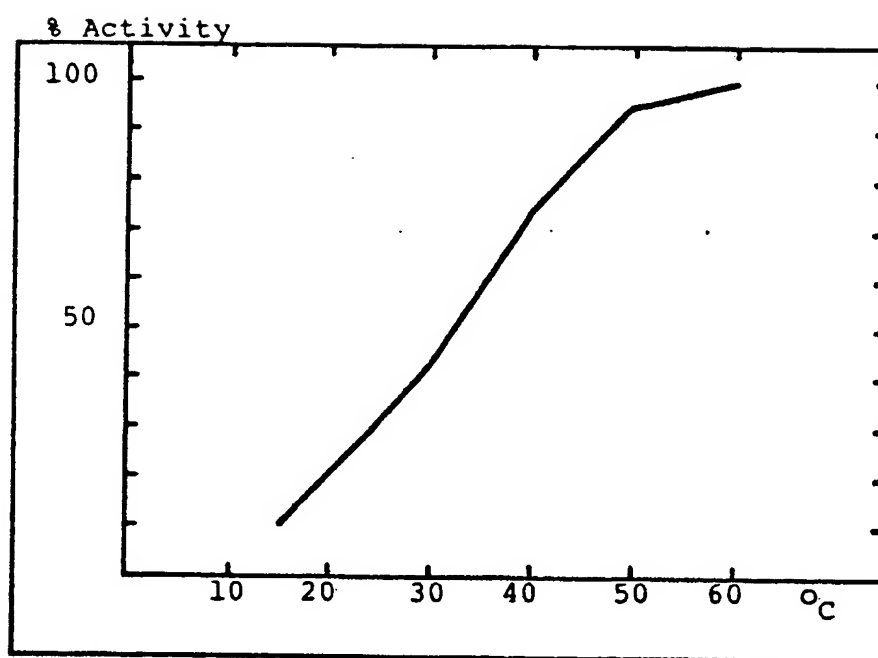


FIG. 4

5/7

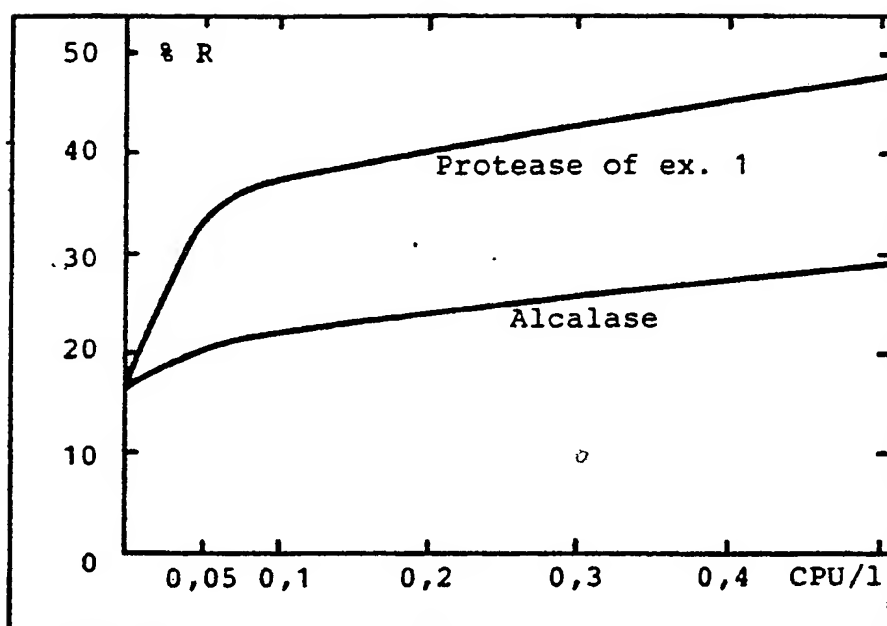


FIG. 5

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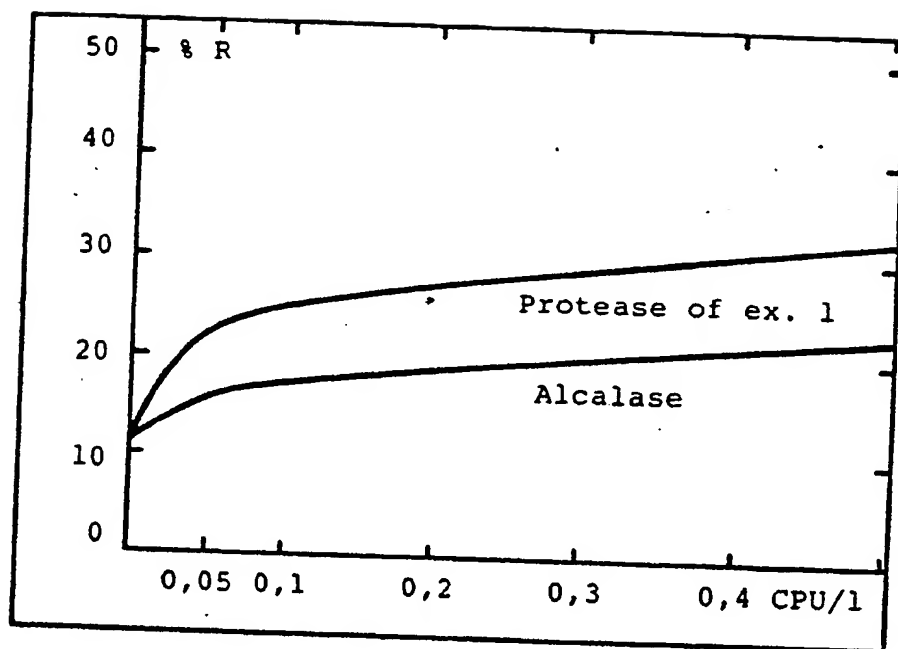


FIG. 6

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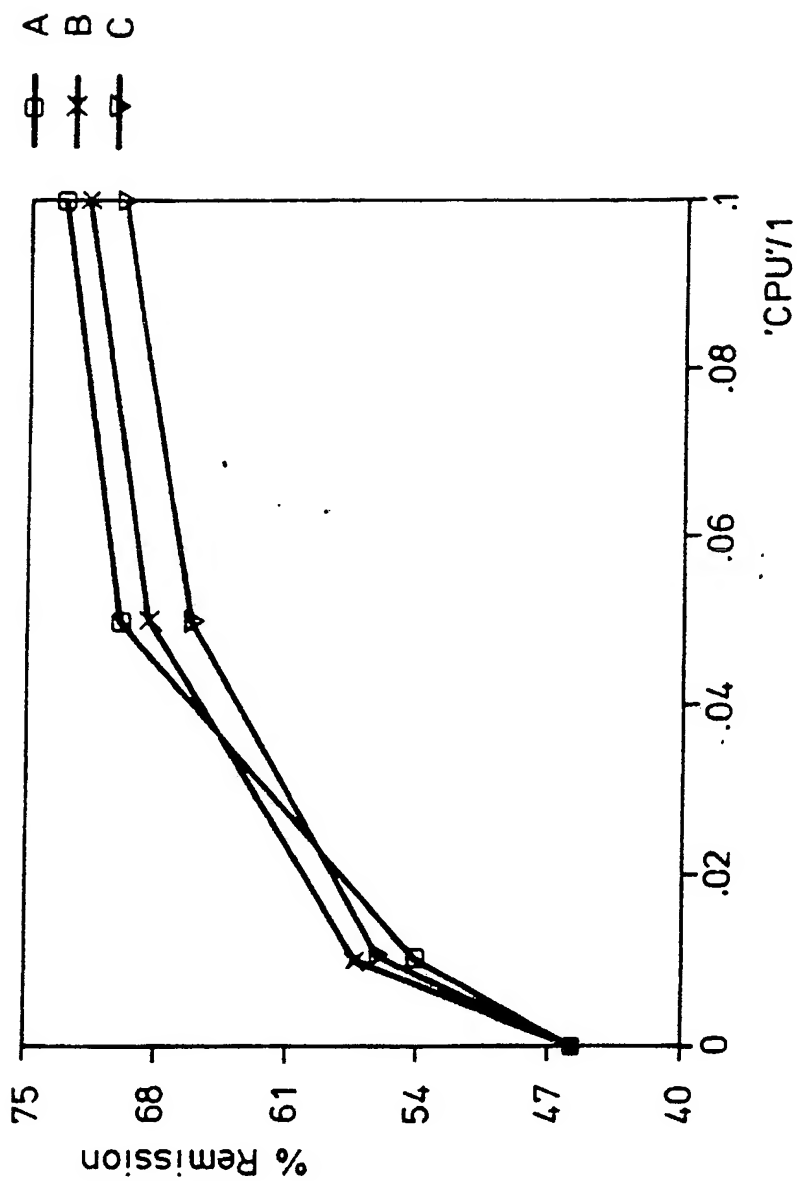



Fig. 7

INTERNATIONAL SEARCH REPORT

PCT/DK87/00101

International Application No

| | | |
|--|---|-------------------------------------|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| C 12 N 9/54, C 11 D 3/386 | | 4 |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁷ | | |
| Classification System | Classification Symbols | |
| IPC 4 | C 12 N 9/54, /56; C 11 D 3/386 | |
| US C1 | 195: 62, 66; 435: 221 | |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ | | |
| SE, NO, DK, FI classes as above | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ | | |
| Category ¹⁰ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| X | GB, A, 1 243 784 (NOVO TERAPEUTISK LABORATORIUM A/S) & NL, 6814166 DE, 1800508 FR, 1587801 BE, 721730 US, 3723250 CH, 538539 CA, 954807 AT, 315331 AT, 299101 GB, 1234445 | 1-10 |
| X | Kirk-Othmer, "Encyclopedia of chemical technology" Volume 9, 1980, John Wiley & Sons, New York, p. 138-147, see p. 140 table 2. | 1-10 |
| X | US, A, 3 827 938 (NOVO TERAPEUTISK LABORATORIUM A/S) 6 August 1974 See column 1, line 28-30. .../... | 1-10 |
| <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report | |
| 1987-10-21 | 1987 -11- 03 | |
| International Searching Authority | Signature of Authorized Officer | |
| Swedish Patent Office |  Yvonne Siösteen | |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|--|----------------------|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| | & NL, 7207050 FR, 2140064 DE, 2224777 GB, 1395895 CH, 599975 | |
| X | US, A, 4 002 572 (TE NIJENHUIS) 11 January 1977 & NL, 7513433 FR, 2292042 LU, 73826 BE, 835693 DE, 2551742 GB, 1519148 JP, 51082783 JP, 51125407 JP, 55148086 US, 30602 | 1-10 |
| X | US, A, 4 052 262 (HORIKOSHI ET AL) 4 October 1977 & NL, 7007854 DE, 2026092 FR, 2052484 GB, 1308238 SE, 368961 | 1-10 |
| X | US, A, 3 748 233 (LEVER BROTHERS COMPANY) 24 July 1973 & NL, 7013286 DE, 2044161 FR, 2061067 GB, 1306867 CH, 542279 BE, 755886 | 1-10 |
| X | US, A, 4 052 262 (HORIKOSHI ET AL) 4 October 1977 & NL, 7007854 DE, 2026092 FR, 2052484 GB, 1308238 SE, 368961 | 1-10 |
| | .../... | |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|--|----------------------|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| X | US, A, 4 480 037 (ICHISHIMA ET AL) 30 October 1984 See table 2. & JP, 58134990 FR, 2521163 AU, 10970/83 GB, 2116561 CA, 1193213 AU, 556045 | 1-10 |
| X | DE, A, 2 005 232 (AJINOMOTO CO. INC) 12 November 1970 & NL, 6919594 FR, 2041793 GB, 1287212 US, 3622459 | 1-10 |
| X | US, A, 3 905 869 (HIDAKA ET AL) 16 September 1975 & FR, 2197569 DE, 2343963 CA, 990672 GB, 1451074 JP, 49042883 | 1-10 |
| X | US, A, 3 674 643 (AUNSTRUP ET AL) 4 July 1972 See column 5, line 15-17. & NL, 6815944 DE, 1807185 GB, 1205403 FR, 1599036 CH, 523319 BE, 723649 AT, 290444 SE, 372561 | 1-10 |
| X | US, A, 3 723 250 (AUNSTRUP ET AL) 27 March 1973 & NL, 6814166 DE, 1800508 FR, 1587801 GB, 1243784 BE, 721730 CH, 538539 CA, 954807 AT, 315331 AT, 299101 GB, 1234445 / | 1-10 |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|--|----------------------|
| Category.* | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| X | Chemical abstracts, Vol 81, 1974, abstract No 59820t, Su, Yuan-Chi et al J. Chin. Biochem. Soc. 1973 2(1), 1-10 (Eng). | 1-10 |
| X | Chemical abstracts, Vol 102, 1985, abstract No 202605m, NL 8302790, 8 Aug 1983. | 1-10 |
| X | Chemical abstracts, Vol 96, 1982, abstract No 64654f, Dambmann et al, Proc. Int. Symp. 1980 (Pub. 1981) 231-44 (Eng). | 1-10 |
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